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Changes in immunological profile as a function of urbanization and lifestyle

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Summary

Differences in lifestyle and break with natural environment appear to be associated with changes in the immune system resulting in various adverse health effects. Although genetics can have a major impact on the immune system and disease susceptibility, the contribution of environmental factors is thought to be substantial. Here, we investigated the immunological profile of healthy volunteers living in a rural and an urban area of a developing African country (Senegal), and in a European country (the Netherlands). Using flow cytometry, we investigated T helper type 1 (Th1), Th2, Th17, Th22 and regulatory T cells, as well as CD4$^+$ T-cell and B-cell activation markers, and subsets of memory T and B cells in the peripheral blood. Rural Senegalese had significantly higher frequencies of Th1, Th2 and Th22 cells, memory CD4$^+$ T and B cells, as well as activated CD4$^+$ T and B cells compared with urban Senegalese and urban Dutch people. Within the Senegalese population, rural paritcipants displayed significantly higher frequencies of Th2 and Th22 cells, as well as higher pro-inflammatory and T-cell activation and memory profiles compared with the urban population. The greater magnitude of immune activation and the enlarged memory pool, together with Th2 polarization, seen in rural participants from Africa, followed by urban Africans and Europeans suggest that environmental changes may define immunological footprints, which could have consequences for disease patterns in general and vaccine responses in particular.

Keywords: immune response differences; rural; urban.

Introduction

In the last decades, a global increase in the prevalence of many chronic inflammatory diseases has been reported in affluent countries.[1,2] Although genetic predisposition has been reported to govern the development of some complex inflammatory disorders,[3–5] it cannot explain the rapid changes seen in the prevalence of these diseases. Environmental conditions such as exposure to multiple infections, as well as sub-optimal nutritional status or traditional lifestyle might affect not only the physiology, but also the immune system. It has been reported that early exposure to harsh environmental conditions with high microbial loads may be a protective factor against inflammatory diseases such as allergies, whilst decreasing exposure to microorganisms as well as the loss of traditional lifestyle and dietary shifts might increase the risk of developing allergic diseases.[6,7] Epidemiological studies have indicated that allergic disorders are highly prevalent in affluent countries and urban centres of developing countries, whereas they are rare in rural communities with a high degree of exposure to microorganisms and parasites.[8–10] Currently, it is thought that changes to the immune system in response to the environment might be responsible for the rising prevalence of inflammatory diseases such as allergies.

The immune system is equipped with different cell types involved in recognition and elimination of a wide variety of microorganisms including CD4$^+$ T cells, which are central to the control of infections and the regulation of immune responses.[11–18] T helper type 1 (Th1) immunological responses are mainly involved in defence against intracellular pathogens,[19] Th2 responses against helminths and ectoparasites,[16] and Th17 cells appear to be important for defence against extracellular bacteria and fungi.[17] However, uncontrolled T-cell responses can cause tissue

and organ damage. For example, overshoot of Th1 and Th17 cells can be associated with autoimmune and inflammatory diseases,[20,21] while Th2 cell over-activation can lead to allergic disorders.[22] Activated immune responses are kept under control by a regulatory network, with regulatory T (Treg) cells at the forefront, which express suppressory molecules, capable of controlling activated effector T cells.[23] Interleukin-22 (IL-22) produced by a distinct Th22 cell subset,[18] or by Th17 cells in combination with IL-17,[24] has been reported in inflammatory diseases.[25,26] Stages of activation of adaptive cells and their development into functional effector and memory cells occurring upon repeated microbial challenges[27,28] might be used to compare the immunological status of people from different environmental settings.

We hypothesize that the different environments resulting from urbanization and the differences in lifestyle impact on the immune system. In this study, we used identical reagents and protocols to investigate the $CD4^+$ T-cell subsets, pro- and anti-inflammatory cytokines, T-cell activation and memory phenotypes, and memory B cells in populations from Europe and within rural and urban areas in Africa.

Materials and methods

Study subjects

A total of 30 healthy volunteers aged from 20 to 30 years was recruited from three different geographical areas: a rural and an urban area in Senegal (West Africa) and an urban area in the Netherlands (western Europe). The rural African subjects ($n = 10$) are farmers living in the village of Pakh in northern Senegal who were recruited as a control group in a study of immune responses underlying the pathology of human schistosomiasis; they were negative for malaria (after thick smear and malaria rapid test), *Schistosoma* species (after Kato–Katz test on faeces and a urine filtration test using 12-μm pore size filters) as well as *Ascaris lumbricoides*, *Trichuris trichiura* and hookworm following microscopic examination of the faeces.[29] The urban population from Africa ($n = 10$) are laboratory personnel of the Aristide Le Dantec university hospital of Dakar, the capital of Senegal, who volunteered to be enrolled in this study.

The largest ethnic group in Senegal is the Wolof community, representing 43·3% of the population and distributed throughout the country but predominantly in the west and north.[30] The second largest community is Pular (23·8%) followed by Serer (14·7%), which is the community the most closely related to Wolof in terms of descendants.[31] The predominant ethnic groups in Senegal all share a common cultural background so that there are no effective cultural barriers between them and marriage between ethnic groups is very common.[32] All the rural Senegalese and nine of the 10 urban Senegalese belonged to the Wolof ethnic group and the one remaining individual from the urban Senegalese group was from the Serer community. Regarding the European participants, they were Dutch students of the Leiden University Medical Centre of the Netherlands ($n = 10$) who volunteered to donate blood. All subjects were interrogated on their health conditions and medical histories by a clinician and none of them presented clinical signs of current infection or history of chronic inflammatory disease. However, all Senegalese individuals reported having malaria at least once in their life.

This study was approved by the 'Comité National d'Ethique de la Recherche en Santé' of Senegal (Permit Number: 0044MSPHP/DS/CNERS). Written informed consent was obtained from all participants.

Cell isolation and fixation

From heparinized venous blood, peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll density gradient centrifugation (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) within 4 hr after blood collection. After isolation, 1×10^6 PBMCs were washed with PBS (Invivogen, Carlsbad, CA), fixed with Transcription factor fixation buffer (eBioscience, San Diego, CA) for 1 hr and frozen in RPMI-1640 (Invitrogen, Carlsbad, CA) containing 10% heat-inactivated fetal bovine serum (FBS) (Invitrogen) and 10% DMSO (Merck, Darmstadt, Germany). The RPMI was supplemented with 100 U/ml penicillin (Gibco, Paisley, UK), 100 U/ml streptomycin (Sigma-Aldrich, St Louis, MO), 1 mM pyruvate (Sigma-Aldrich) and 2 mM glutamate (Sigma-Aldrich).

Cell stimulation for further intracellular cytokine staining

To assess T-cell cytokines, 1×10^6 PBMCs were stimulated for 6 hr with 100 ng/ml PMA (Sigma-Aldrich) and 1 μg/ml Ionomycin (Sigma-Aldrich). After 2 hr, 10 μg/ml brefeldin A (Sigma-Aldrich) was added and cells were incubated for four more hours at 37° under 5% CO_2. Stimulated cells were fixed with 2% cold fresh-made formaldehyde solution (Sigma-Aldrich) in PBS for 15 min, washed twice with PBS and then frozen in 10% FBS/10% DMSO/RPMI freezing medium.

Flow cytometric analysis

The fixed and cryopreserved PBMCs (stimulated and unstimulated) from Senegal were shipped on dry ice (Air-Liquid) to Leiden, the Netherlands. There, both the Senegalese and Dutch unstimulated and PMA/ionomycin-stimulated PBMCs were thawed and washed once with 10% FBS/RPMI and once with PBS. *Ex vivo* cells fixed with eBioscience Transcription factor fixation buffer (for measurement of transcription factors, activation and memory

markers) were permeabilized with eBioscience Transcription factor permeabilization buffer (eBioscience) for 5 min at room temperature, while cells stimulated with PMA and ionomycin and fixed with formaldehyde (for intracellular cytokine assessment) were permeabilized with 0·5% saponin (Sigma-Aldrich). Subsequently, 25×10^4 *ex vivo*-fixed PBMCs were stained with three different panels. Panel 1: AmCyam-labelled anti-CD3 (BD Biosciences, San Jose, CA), phycoerythrin (PE)-Cy7-labelled anti-CD4 (BD Biosciences), PE-labelled anti-ROR-γt (eBioscience), Peridinin chlorophyll protein (PerCP)-Cy5.5-labelled anti-T-bet (eBioscience), and eFluor660-labelled anti-GATA-3 (eBioscience); Panel 2: allophycocyanin (APC)-labelled anti-CD3 (BD Biosciences), PerCP-labelled anti-CD4 (BD Biosciences), PE-Cy7-labelled anti-CD25 (BD Biosciences), FITC-labelled anti-FOXP3 (eBioscience), PE-labelled anti-CD127 (BD Biosciences); Panel 3: PE-Cy7-labelled anti-CD4 (BD Biosciences), FITC-labelled anti-CD19 (BD Biosciences), PerCP-labelled anti-CD14 (BD Biosciences), PE-labelled anti-CD86 (BD Biosciences), APC-eFluor780-labelled anti-HLA-DR (eBioscience), APC-labelled anti-CD27 (BD Biosciences), and PB-labelled anti-CD45RO (Biolegend, San Diego, CA). To assess intracellular cytokines, 25×10^4 PBMCs were stained using two different panels. Panel 4: AmCyan-labelled anti-CD3 (BD Biosciences), PerCP-labelled anti-CD4 (BD Biosciences), AlexaFluor488-labelled anti-IL-17 (eBioscience), PE-labelled anti-IL-22 (R& D Systems, Minneapolis, MN), PE-Cy7-labelled anti-IL-4 (eBioscience), APC-labelled anti-interferon-γ (IFN-γ; BD Biosciences); Panel 5: AmCyan-labelled anti-CD3 (BD Biosciences), PerCP-labelled anti-CD4 (BD Biosciences), PE-labelled anti-IL-10 (BD Biosciences), FITC-labelled anti-IL-2 (BD Biosciences), PE-Cy7-labelled anti-tumour necrosis factor-α (TNF-α; eBioscience). All the staining buffers contained anti-Fcγ receptor (FcγR) (eBioscience) to prevent non-specific bindings. Staining was performed for 30 min at 4°; the stained cells were then washed and resuspended in PBS supplemented with 0·5% BSA (Sigma-Aldrich) and 2 mM EDTA (Sigma-Aldrich), and acquired with FACSCanto II flow cytometer (BD Biosciences). Results were analysed by FlowJo for MAC version 9.8.6 (Tree Star Inc., Ashland, OR). Positions of gates were confirmed with 'fluorescence-minus-one' controls. The characteristics of the monoclonal antibodies used for the flow cytometric analysis are shown in Table S1.

Statistical analysis

Data were analysed with SPSS Statistics for Windows, version 17 (IBM Inc., Armonk, NY). GraphPad Prism version 5.00 for Windows (GraphPad Software Inc., San Diego, CA) and SPSS version 20 for Windows (IBM Inc.) were used for graphing. Differences between groups were evaluated using the chi-square test and the non-parametric Mann–Whitney *U*-test. The Kruskal–Wallis *H*-test was used for multiple comparisons, followed by the Mann–Witney *U*-test for post hoc analysis. The correlations between cytokines were calculated using the non-parametric Spearman Rho test. The level of significance for all statistical tests was set at $P < 0{\cdot}05$.

Results

Study population

Peripheral blood was collected from healthy volunteers aged from 20 to 30 years and distributed in three groups: (i) Rural subjects from Senegal (Ru-Sen), (median age 27 years; min. 20, max. 30; 50% male); (ii) urban individuals from Senegal (Ur-Sen) (median age 27 years; min. 26, max. 30; 50% male); and (iii) urban subjects form the Netherlands (Ur-Dut) (median age 26·5 years; min. 26, max. 29; 40% male). Regarding the ethnic relatedness of the Senegalese study groups, all the rural Senegalese belonged to the Wolof ethnic group while among the urban Senegalese subjects, nine of 10 were Wolof and one of 10 was Serer. Age and sex did not significantly differ between groups ($P = 0{\cdot}469$ and 0·465 respectively).

Distribution of $CD4^+$ T-cell subsets, pro- and anti-inflammatory $CD4^+$ T-cell cytokines in rural and urban populations

To compare the adaptive immune system between rural and urban populations, the relative proportions of $CD4^+$ T-cell subsets were compared between rural Senegalese, urban Senegalese and urban Dutch people. The subsets were compared based on transcription factor expression *ex vivo* [T-box expressed in T cells (T-bet), GATA binding protein 3 (GATA-3), retinoid-related orphan receptor γt (ROR-γt), and forkhead box P3 (FOXP3)] as well as intracellular cytokine production (IFN-γ, IL-4, IL-17, IL-22, IL-2, TNF-α and IL-10) in response to PMA and ionomycin stimulation in isolated PBMCs. The gating strategies for transcription factors and intracellular cytokines are shown in the Supporting information (Figs S1 and S2). Th1 cells were defined as T-bet$^+$ or IFN-γ$^+$ $CD4^+$ T cells, Th2 cells as GATA-3$^+$ or IL-4$^+$ $CD4^+$ T cells and Th17 cells as ROR-γt$^+$ or IL-17$^+$ $CD4^+$ T cells. For all three subsets, the highest percentages of transcription factor or cytokine-positive $CD4^+$ T cells were found in the rural Senegalese group, with intermediate percentages in urban Senegalese and the lowest percentages in the Dutch group (Fig. 1a,b). However, ROR-γt$^+$ $CD4^+$ T cells were higher in the Dutch group compared with the urban Senegalese group. Furthermore, as both Th1 and Th2 percentages decreased from urban to rural setting, the Th2 : Th1 ratio was assessed. The IL-4 : IFN-γ ratio was lower in the Dutch group (Fig. 1c), pointing to a Th2 bias in rural Senegalese.

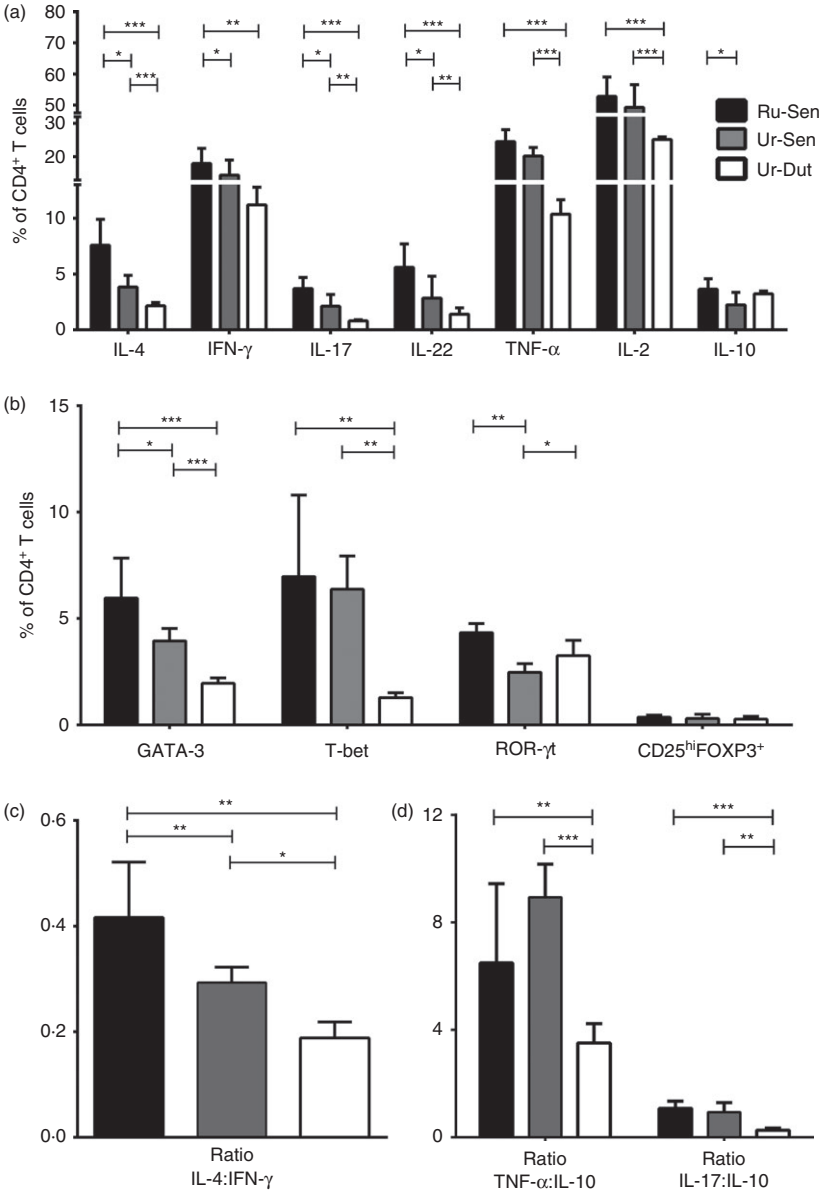

Figure 1. Distribution of $CD4^+$ T-cell subsets, pro-inflammatory and anti-inflammatory $CD4^+$ T-cell cytokines in rural and urban populations. Percentages of (a) interleukin-4-positive ($IL\text{-}4^+$), interferon-γ-positive ($IFN\text{-}\gamma^+$), $IL\text{-}17^+$, $IL\text{-}22^+$, tumour necrosis factor-α-positive ($TNF\text{-}\alpha^+$), $IL\text{-}2^+$, and $IL\text{-}10^+$ cells in total $CD4^+$ T cells assessed in PMA and ionomycin-stimulated peripheral blood mononuclear cells (PBMCs), (b) GATA-3^+ (GATA binding protein 3), T-bet^+ (T-box expressed in T cells), ROR-γt^+ (retinoid-related orphan receptor γt), and $CD25^{hi}$ $FOXP3^+$ cells in total $CD4^+$ T cells that have been assessed in *ex vivo* PBMCs, and the ratios (c) IL-4 to INF-γ, (d) TNF-α to IL-10 and IL-17 to IL-10 in the rural Senegalese (Ru-Sen; $n = 10$), urban Senegalese (Ur-Sen; $n = 10$), and urban Dutch (Ur-Dut; $n = 10$) groups are shown. Data are shown as median values and 75% interquartiles. *P*-values were calculated in SPSS 17 using non-parametric Mann–Whitney *U*-test and the graphing was performed using GRAPHPAD PRISM version 5.00 for Windows. Only *P*-values for significant differences are shown in the figures. $*P < 0{\cdot}05$, $**P < 0{\cdot}01$, $***P < 0{\cdot}001$.

Next to the Th1, Th2 and Th17 cells, general pro-inflammatory and anti-inflammatory cytokine expression by $CD4^+$ T cells was analysed, as well as percentage of $CD25^{hi}$ $FOXP3^+$ Treg cells. The percentages of IL-2, TNF-α and IL-22 were higher in the rural Senegalese group compared with both urban groups. Among Senegalese groups, the percentages of TNF-α and IL-2 were higher in the rural group compared with the urban group but the difference did not reach statistical significance. For $IL\text{-}10^+$ $CD4^+$ T cells, a difference between urban and rural Senegalese was observed, whereas no significant difference was observed between Senegalese and Europeans; no differences were seen in $CD25^{hi}$ $FOXP3^+$ Treg cells between the three groups.

To further evaluate the differences in pro-inflammatory and anti-inflammatory cytokines, two pro/anti-inflammatory ratios were compared between the rural and urban populations. Both the TNF-α : IL-10 and IL-17 : IL-10 ratios pointed towards a more pro-inflammatory profile for Senegalese compared with Dutch people, whereas rural and urban Senegalese groups did not differ significantly (Fig. 1d).

IL-10 is correlated with IFN-γ, IL-17 and IL-4 in rural but not in urban populations

Correlations between percentages of IL-10-producing $CD4^+$ T cells and Th1 ($IFN\text{-}\gamma^+$), Th2 ($IL\text{-}4^+$) and Th17 ($IL\text{-}17^+$) cells were assessed in each of the study groups. Interleukin-10 was positively correlated with IFN-γ, IL-17 and IL-4 in the rural group. Interestingly, no significant correlations were found in either the urban group from Senegal or in the Dutch group (Fig. 2).

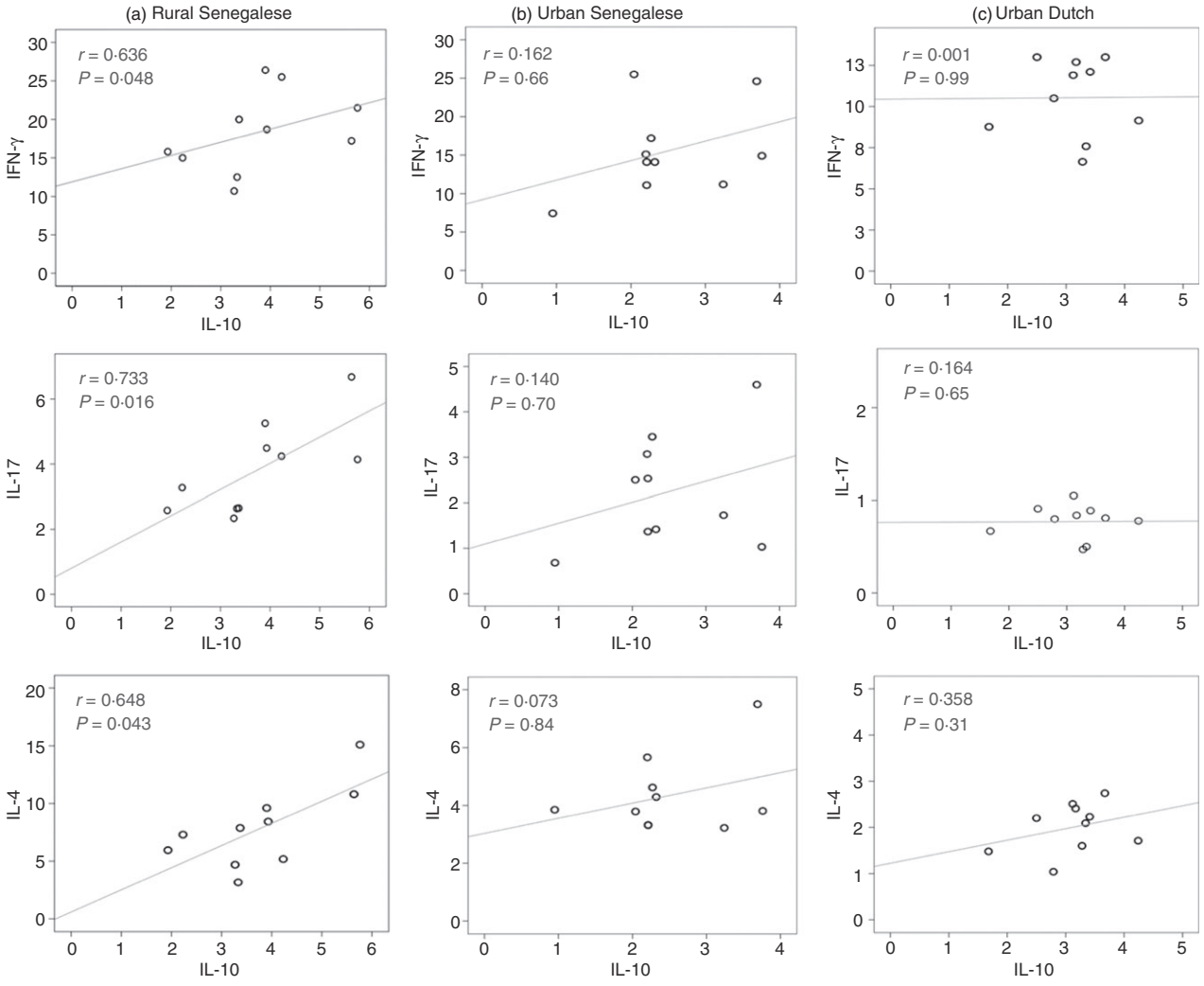

Figure 2. Interleukin-10 (IL-10) is positively correlated with interferon-γ (IFN-γ), IL-17 and IL-4 in rural subjects but not urban populations. Scatter plots assessing correlation between IL-10 and IFN-γ, IL-17, and IL-4 in the (a) rural Senegalese (Ru-Sen; $n = 10$), (b) urban Senegalese (Ur-Sen; $n = 10$), and (c) urban Dutch (Ur-Dut; $n = 10$) groups. Correlations were calculated in SPSS 20 using Spearman Rho test and the graphing was performed using SPSS 20. r, coefficient of correlation.

Memory CD4$^+$ T cells and B cells as rural to urban gradient

We investigated memory CD4$^+$ T cells and B cells (gating strategies of subsets of memory T and B cells are shown in the Supporting information; Fig. S3). The frequency of CD45RO$^+$ memory CD4$^+$ T cells was significantly lower in the Dutch group compared with the urban Senegalese and rural Senegalese groups (Fig. 3a). Urban individuals in Senegal displayed higher levels of CD4$^+$ CD45RO$^+$ memory T cells compared with rural subjects from Senegal.

Beside total memory cells, we defined effector memory CD4$^+$ T cells as CD45RO$^+$ CD27$^-$ CD4$^+$ T cells and central memory CD4$^+$ T cells as CD45RO$^+$ CD27$^+$ CD4$^+$ T cells.[33] Similarly to total memory cells, frequencies of central memory and effector memory CD4$^+$ T cells were lower in the Dutch group compared with both Senegalese groups; among Senegalese, both central and effector memory cells appeared higher in the rural group compared with the urban group (Fig. 3b).

With regard to B cells, we similarly found a significantly lower percentage of CD27$^+$ memory B cells in the Dutch group compared with the Senegalese groups (Fig. 4a).

Senegalese subjects display higher frequencies of memory CD4$^+$ T cells and B cells compared with Dutch people

CD86 and HLA-DR, expressed on lymphocytes upon activation,[11,34] were investigated; the gating strategies of CD4$^+$ T and B cells expressing these CD86 and/or HLA-DR

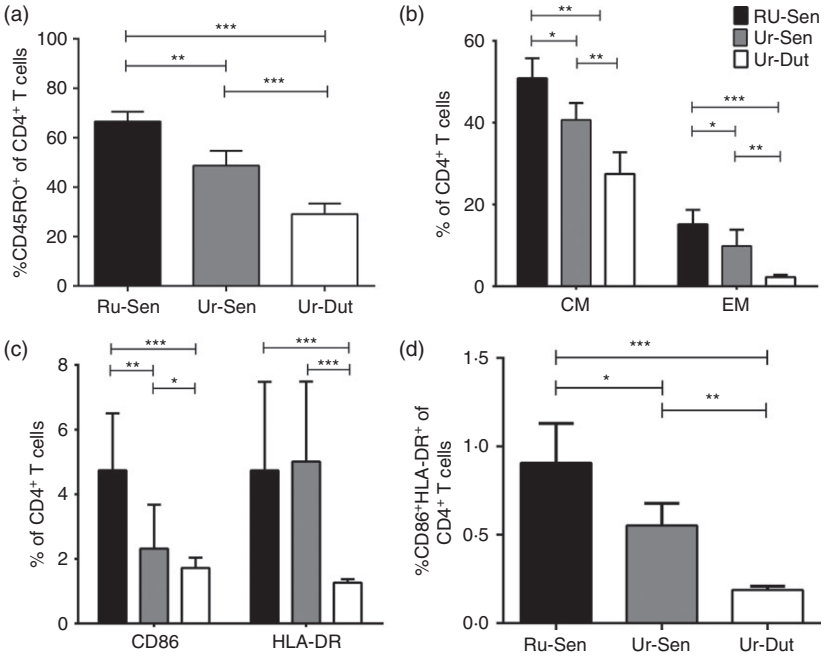

Figure 3. Expression of memory phenotypes and activation markers of $CD4^+$ T cells as rural to urban gradient. Percentages of (a) $CD45RO^+$, (b) $CD45RO^+$ $CD27^+$ central memory (CM) and $CD45RO^+$ $CD27^-$ effector memory (EM), (c) $CD86^+$ and $HLA\text{-}DR^+$, and (d) $CD86^+$ $HLA\text{-}DR^+$ in $CD4^+$ T cells the rural Senegalese (Ru-Sen; $n = 10$), urban Senegalese (Ur-Sen; $n = 10$), and urban Dutch (Ur-Dut; $n = 10$) groups are shown. *P*-values were calculated in SPSS 17 using non-parametric Mann–Whitney *U*-test and the graphing was performed using GRAPHPAD PRISM version 5.00 for Windows. Only *P*-values for significant differences are shown in the figures. $^*P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$, $^{***}P < 0{\cdot}001$.

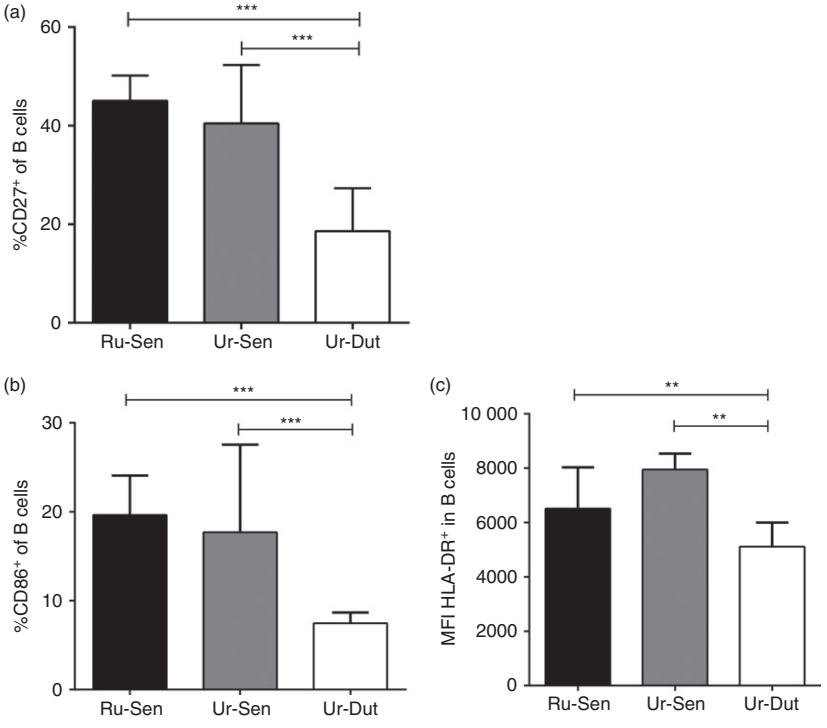

Figure 4. Expression of memory phenotype and activation markers of B cells as rural to urban gradient. Percentages of (a) $CD27^+$, (b) $CD86^+$, and (c) mean fluorescence intensity (MFI) of HLA-DR in B cells in the rural Senegalese (Ru-Sen; $n = 10$), urban Senegalese (Ur-Sen; $n = 10$), and urban Dutch (Ur-Dut; $n = 10$) groups are shown. *P*-values were calculated in SPSS 17 using non-parametric Mann–Whitney *U*-test and the graphing was performed using GRAPHPAD PRISM version 5.00 for Windows. Only *P*-values for significant differences are shown in the figures. $^{**}P < 0{\cdot}01$, $^{***}P < 0{\cdot}001$.

are shown in the Supporting information (Fig. S4). The percentage of $CD86^+$ $CD4^+$ T cells in the Dutch group was lower than in the Senegalese groups. Among Senegalese groups, the percentages of $CD86^+$ $CD4^+$ T cells were higher in rural subjects compared with urban subjects (Fig. 3c). The percentage of $HLA\text{-}DR^+$ $CD4^+$ T cells was also lower in the Dutch group compared with urban and rural people from Senegal. In contrast to CD86, the percentage of HLA-DR-expressing $CD4^+$ T cells did differ between Senegalese subjects. Furthermore, the percentage of $CD4^+$ T cells expressing both CD86 and HLA-DR decreased from rural via urban Senegalese to the lowest value for Dutch subjects (Fig. 3d).

With regard to B-cell activation markers, the percentage of $CD86^+$ B cells was lower in the urban Dutch group compared with the urban and rural Senegalese groups

(Fig. 4b). Furthermore, the mean fluorescence intensity of HLA-DR$^+$ B cells was lower in the Dutch group compared with the urban and rural Senegalese people (Fig. 4c). However, neither the percentage of CD86-expressing nor that of HLA-DR-expressing CD19$^+$ B cells differed between Senegalese subjects.

Discussion

We investigated the immunological profile of healthy adults from rural and urban settings in Africa (Senegal) and urban Dutch subjects (the Netherlands). All the rural as well as nine of 10 of the Senegalese urban subjects belonged to the Wolof ethnic group, while one urban Senegalese subject belonged to the Serer community, which is the most closely related to the Wolof community in terms of descendants.[31] In these subjects, both *ex vivo* and stimulated cells were analysed to study the immune status. Our results show marked geographical differences in the magnitude and quality of CD4$^+$ T-cell responses.

In fact, the frequencies of Th1 (CD4$^+$ T-bet$^+$ T or CD4$^+$ IFN-γ^+ T) and Th2 cells (CD4$^+$ GATA-3$^+$ T or CD4$^+$ IL-4$^+$ T cells) as well as pro-inflammatory profiles (elevated level of TNF-α, IL-17, ratio TNF-α : IL-10 and ratio IL-17 : IL-10) appear greater in rural Senegalese followed by urban Senegalese and urban Dutch. The rural-to-urban gradient in these pro-inflammatory profiles supports the finding that populations living in hostile tropical environments display overall greater pro-inflammatory responses than populations living in a temperate environment.[35] Our results are consistent with the finding of Roetynck *et al.*[36] showing that African subjects from a rural community in Kenya displayed a greater overall CD4$^+$ T-cell cytokine response compared with African and European individuals from urban settings. The finding that urban Senegalese display a lower pro-inflammatory profile compared with rural subjects suggests that immunological changes might be associated with a break from traditional lifestyle and lower exposure to infections. This is supported by the reports of changes in immunological profiles of African migrants in Europe after several years.[37,38] More recently, Smolen *et al.*[39] have shown, in 2-year-old infants, that cytokine and chemokine production in response to pattern recognition receptor ligands were lower in South African infants compared with Europeans, North Americans and South American infants. In terms of immune responsiveness between low (in Africa) and high (in Europe) income countries, our results are different as we report, if any, a higher immunological response in Senegalese. This difference might be a result of the age of the study participants (2 years versus young adults), because it has been reported that differences in African and European immune responses are age-dependent;[40] but more importantly, South African infants had received measles vaccinations at 18 months of age whereas this was not the case in the other countries, which had different vaccination schedules. Indeed, vaccination can have a profound effect on the general immunological responsiveness, both short and long term.[41]

It is known that high Th2 and Th17 responses and pro-inflammatory responses in general, are linked to allergies and inflammatory diseases.[20,21] So an important question to address is why inflammatory and autoimmune diseases remain relatively rare in rural settings where higher Th2 and Th17 responses are found. A possible explanation would be that whereas IL-10 significantly correlates with IFN-γ, IL-17 and IL-4 in the rural setting, this is not the case in either urban Dutch or urban Senegalese study subjects. This indicates that the high Th2 and Th17 cytokine levels observed in rural subjects is accompanied by high regulatory-IL-10 cytokine production, suggesting that there might be more modified responses in rural populations.[42] However, it is also possible that correlations were not significant in urban subjects because of the lower cytokine responses in urban compared with rural populations. Percentages of IL-17$^+$ CD4$^+$ T cells decreased gradually from rural Senegalese, via urban Senegalese to Dutch participants; however, this was not seen when examining ROR-γt. While rural and urban Senegalese still followed the same pattern, urban Dutch had an increased percentage of ROR-γt$^+$ T cells compared with urban Senegalese. This discrepancy between Th17 transcription factor and cytokine expression needs to be confirmed and further investigated.

HLA-DR and B7-2 co-stimulatory molecules (including CD86) constitutively expressed antigen-presenting cells have been shown to be up-regulated on T cells[11,13,14,43] and B cells[44,45] upon repeated microbial challenge. We found that frequencies of T and B cells expressing HLA-DR and CD86 also follow a rural-to-urban gradient, highlighting the higher immune activation in rural subjects as compared with the urban group in Senegal and the more activated immune system in Senegalese compared with the Dutch group. This is in line with the study of Kemp *et al.*[46] comparing Ghanaians (living in an area with high prevalence of malaria, mycobacteria, Epstein–Barr virus) and European counterparts, reporting elevated frequencies of cytokine-producing cells in Ghanaians, which was correlated with the frequencies of activated cells. However, in light of the greater cell activation and magnitude of the CD4$^+$ T-cell response we found in rural settings, our results do not support the notion that persistent immune activation in developing countries leads to T-cell hyporesponsiveness.[47,48] However, most studies reporting immune hyporesponsiveness have assessed the *in vitro* immune responses after antigenic challenge and not *ex vivo* or stimulated with PMA and ionomycin, as we have.

In addition to the cytokine profiles and activation status, we also investigated the memory phenotypes showing that Senegalese people, especially rural subjects, display

more CD45RO$^+$ memory CD4$^+$ T cells and CD27$^+$ B cells compared with the Dutch group. This higher level of memory cells in rural subjects is in line with the finding that environment impacts on the immune system.[37,49] Next to the total memory phenotype, we also defined CD45RO$^+$ CD27$^+$ central memory CD4$^+$ T cells and CD45RO$^+$ CD27$^-$ effector memory CD4$^+$ T cells as in the study of Kovacs *et al.*[50] Both frequencies of central memory and effector memory CD4$^+$ T cells were lower in Europeans compared with both rural and urban groups in Africa. Highly differentiated populations of effector memory cells have been shown to accumulate in people having long-term activation of their immune system, such as patients with persistent viral infections[51] and those with chronic inflammatory syndromes.[52,53] These findings along with the findings of increased cell activation are again in line with the greater immune activation observed in rural compared with urban subjects.

With regard to these immunological differences between Senegalese and Dutch subjects and within Senegalese people living in rural and urban settings, our findings suggest that geographical differences might define immunological footprints. This is supported by Idaghdour *et al.* who showed in Moroccans from the same tribe living in three distinct areas (urban area, rural mountainous area and desert area), that up to 30% of the leucocyte transcriptome was associated with geographical differences.[54] Furthermore, variation in immunological profiles attributed to a particular geographical setting might have an impact, at least partly, on the pathogenesis and spatial distribution of certain chronic inflammatory diseases. As the immune response is central to the success of a vaccine, our findings along with others highlighting strong geographical and environmental differences in the immune system, indicate that this needs to form an integral part of vaccine development for optimal results.

Although geographical differences impact on the immune system, further investigations into the role that nutrition, genetics, epigenetics and infectious diseases would help to better understand the various adverse health effects associated with changes in our immune system and susceptibility to diseases.

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Disclosures

The authors declare no financial or commercial conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Characteristics of the monoclonal antibodies used for the flow cytometric analysis.

Figure S1. Gating strategy of T helper type 1 (Th1) and Th2 transcription factors and intracellular cytokines.

Figure S2. Gating strategy of regulatory T (Treg) cells and T helper type 17 (Th17) cells.

Figure S3. Gating strategy of $CD4^+$ T-cell and B-cell memory cells.

Figure S4. Gaiting strategy of $CD4^+$ T-cell and B-cell activation markers.